Acceleration of Tensile Strength of Incisions Treated with EGF and TGF-β

GREGORY L. BROWN, M.D., LUKE J. CURTSINGER, M.D.,* MICHAEL WHITE, M.D., ROBERT O. MITCHELL, M.S., JAMES PIETSCH, B.A., ROBERT NORDQUIST, PH.D.,† ANTHONY VON FRAUNHOFER, PH.D.,‡ and GREGORY S. SCHULTZ, PH.D.

The ability of surgeons to accelerate wound healing through pharmacologic intervention is limited. The effects of locally applied, biosynthetic human epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β) on tensile strength of experimental incisions were investigated. A single dose of EGF in saline failed to increase tensile strength over controls. Thus, EGF was incorporated into multilamellar liposomes, which prolonged the exposure of incisions to EGF ($p < 0.001$). A single dose of EGF in multilamellar liposomes produced a 200% increase in wound tensile strength over controls between 7 and 14 days ($p < 0.05$). Light and electron microscopy of the wounds revealed increased collagen formation and fibroblast proliferation. A single dose of TGB-β in a collagen vehicle stimulated a 51% increase in wound tensile strength at 9 days ($p < 0.01$). We conclude that addition of EGF and TGF-β in appropriate vehicles stimulates early transient increases in wound tensile strength in normal rats.

HEALING OF INCISIONS is a complex, dynamic process, which is characterized by the integrated actions of different cells. The process of wound healing has been separated into broad general phases of inflammation, cellular proliferation, and collagen synthesis, followed by collagen remodeling and maturation. Recent data suggest that these cellular processes are controlled by autocrine or paracrine factors or both, including platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF), and epidermal growth factor (EGF), which are released in the area of the wound by various cell types.[1-3]

From the Price Institute of Surgical Research, Divisions of General and Plastic Surgery, Department of Surgery, Department of Biochemistry, and Department of Restorative Dentistry,‡ University of Louisville, Louisville, Kentucky, and McGee Eye Institute,† Norman, Oklahoma

Several defined peptide growth factors, including EGF, PDGF, FGF, and TGF-β, have been shown to stimulate cellular proliferation and synthesis of the extracellular matrix[4-8] and have been evaluated with various animal models of wound healing. Repeated topical application of EGF accelerated epithelial regeneration of primate corneas[9] and epidermal regeneration in pigs[10] and increased tensile strength of unsutured corneal incisions.[9,11] Also, repeated injections of EGF or prolonged release of EGF from inert polymers elevated the level of DNA and extracellular matrix in cellulose sponges implanted under the skin of rats.[12,13] A single subcutaneous injection of TGF-β induced transient fibrosis *in vivo*, and TGF-β stimulated collagen synthesis by rat kidney fibroblasts *in vitro*.[4]

Locally applied growth factors, which enhance natural incisional healing, could be an important addition to the surgeon's armamentarium for patient care. Therefore, we investigated the effects of EGF and TGF-β on tensile strength of sutured incisions on the backs of normal rats.

Materials and Methods

Materials

Biosynthetic human EGF was provided by Chiron Corporation (Emeryville, CA) and was synthesized by cloned yeast cells transfected with a vector containing a synthetic gene encoding the sequence of human EGF.[14] EGF was purified by high-pressure liquid chromatography

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Reprint requests and correspondence: Gregory S. Schultz, Ph.D., Department of Biochemistry, University of Louisville, Louisville, KY 40292.

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(HPLC), and biologic activity was confirmed by stimulation of DNA synthesis in 3T3 fibroblasts.[15] Biosynthetic human TGF-β was provided by Genentech (South San Francisco, CA), and biologic activity was confirmed by soft agar assay.[16] Lipids and solvents utilized in production of liposomes and other chemicals were from Sigma Chemical Co. (St. Louis, MO). Multilamellar lecithin liposomes and single lamellar liposomes (phosphatidylglycerol, phosphatidylcholine, and cholesterol [1:4:5]) were prepared as described previously.[17,18] The amount of EGF entrapped in liposomes (10 μg/ml) was calculated by measuring the amount of a trace ^{125}I-EGF present in the purified liposomes.

General Wound Model

Adult male Sprague-Dawley rats weighing 230–250 g were anesthetized with ketamine and xylazine and shaved. A single 5-cm interscapular incision penetrating the panniculus carnosus was created in the dorsal midline. Test solutions were placed in the base of incisions, which were then closed with five evenly placed interrupted horizontal mattress sutures of 5–0 Ethilon (Ethicon, Inc., Somerville, NJ). Rats were housed in individual cages with unrestricted food and water. At predetermined intervals after surgery, rats were sacrificed by carbon dioxide inhalation, and the entire incision, including surrounding nonwounded skin, was excised, the panniculus carnosus removed, and the tissue immediately placed on ice-cooled dishes until tested for tensile strength. From each incision, three test strips approximately 5 mm wide were cut perpendicular to the original incision, one from the middle and one from each end. Tensile strength was measured with a Unite-O-Matic FM-20 tensometer (United Calibration Group, Garden Grove, CA) at a velocity of 20 mm/minute and was expressed as Kg/cm^2 (wound tear strength). Mean and standard deviation values were calculated for each experimental condition. The data were compared with one-way analysis of variance (ANOVA), and where differences existed, these were identified by means of Student's *t*-test or the paired *t*-test where appropriate. All animal experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee of the University of Louisville.

Histology

Specimens were placed in 10% neutral buffered formalin, processed through paraffin, sectioned, and stained with hematoxylin and eosin. Specimens for electron microscopy were fixed in cacodylate-buffered 2% glutaraldehyde, embedded in epoxy resin, sectioned and stained with uranyl acetate and lead citrate, then examined with a H-500 electron microscope.

Incisions Treated with Single Doses of EGF Formulated in Saline or Hydrogel

A single interscapular incision was created on the back of 33 rats as described above, and the rats were divided into three treatment groups. One group received 1 ml of phosphate-buffered saline (PBS) containing 10 μg of EGF, one group received 1 ml of PBS alone, and one group was untreated. Tensile strengths were measured 10 days after surgery as described above.

In a similar experiment, single incisions were created on the back of 60 rats, and the rats were divided into three groups. One group of incisions was treated with 1 ml of hydrogel (KY Jelly, Johnson and Johnson, New Brunswick, NJ) containing 10 μg of EGF, one group received 1 ml of hydrogel alone, and a third group was untreated. Tensile strengths of rats from each group were measured at 7, 14, 21, and 28 days and compared by one-way ANOVA.

Incisions Treated with Repeated Doses of EGF in Saline

Interscapular incisions were created as described on the backs of 22 rats. An 18-gauge perforated intravenous catheter was placed in the base of the incision and brought out through a separate stab incision at the base of the neck. Incisions of 11 rats were treated 3 times per day for 5 days by rapidly injecting 500 μl of PBS containing 50 μg of EGF into the catheter, while 11 control rats received injections of PBS. Six days after surgery, catheters were removed from all rats. Seven and 14 days after surgery, rats were killed and tensile strength was measured as described and compared using one way ANOVA and Student's *t*-test.

Stability of Liposome-Encapsulated EGF

Multilamellar lecithin liposomes were prepared containing ^{125}I-EGF together with insulin as a carrier protein. One-milliliter aliquots containing approximately 20,000 cpm were placed inside dialysis tubing with molecular weight cut off at 20,000 daltons. Samples were then dialyzed against 1 L of PBS containing 0.002% sodium azide at either 37 C or 4 C. At daily intervals, each dialysis bag was removed and the amount of ^{125}I-EGF remaining in the bags was measured by gamma scintillation counter. Each bag was then placed in 1 L of fresh PBS solution and dialysis continued. In addition, multilamellar liposomes containing EGF were dialyzed at 37 C against a solution approximating interstitial fluid, which was prepared by diluting plasma threefold with PBS containing 0.002% sodium azide.

TABLE 1. *Tensile Strength of Incisions Treated with a Single Dose of EGF*

	7 Days	14 Days	21 Days	28 Days
EGF-hydrogel	1.69 ± 0.15 (15)	4.10 ± 0.34 (15)	11.01 ± 0.79 (15)	22.81 ± 1.68 (14)
Hydrogel	1.43 ± 0.16 (13)	4.78 ± 0.41 (15)	13.46 ± 1.50 (14)	22.21 ± 2.32 (14)
Untreated	2.42 ± 0.21 (10)	4.87 ± 0.36 (14)	12.22 ± 1.20 (13)	23.10 ± 1.92 (14)

Each incision received 1 mL of hydrogel containing 10 μg of EGF, 1 mL of hydrogel alone, or was untreated. Values are the mean ± standard error of the mean expressed as kg/cm^2; n for each group is expressed in parentheses.

Retention of EGF in Incisions

A single incision was created on the back of 48 rats as before. The rats were divided into four equal groups, and each group received ^{125}I-EGF and insulin carrier protein combined with PBS, 1% hyaluronic acid, multilamellar liposomes, or single lamellar liposomes. One milliliter of a test solution was placed in the base an incision and then sutured as described. At daily intervals, three rats from each of the four groups were sacrificed, and incisions with 3 mm of surrounding nonwounded skin were excised. Residual ^{125}I-EGF was measured with a Beckman 4000 gamma scintillation counter (Beckman Instruments, Palo Alto, CA) and expressed as a percentage of total EGF applied in each incision. At each time point, the average amount of EGF retained in the incision for each formulation was calculated. The data were compared using one way ANOVA and Student's *t*-test.

Incisions Treated with Liposome-Encapsulated EGF

Two separate experiments were performed with EGF formulated in multilamellar liposomes.[17]

In the initial experiment, a single, interscapular incision was created on the backs of 36 rats as described above. The rats were divided into three equal groups. One group received 1 ml of PBS containing 10 μg EGF encapsulated in multilamellar liposomes, a second group received 1 ml of PBS with liposomes containing no EGF, and a third group received 1 ml of PBS alone. At 7, 14, 21, and 28 days after surgery, rats were killed, and triplicate tensile strength measurements were obtained. Tensometer velocity was set at 5 mm/minute.

In the second experiment, 72 rats were divided into three equal groups and treated as described. Rats were killed at 7, 14, 21, and 28 days, and triplicate tensile strength measurements were obtained from each rat, with tensometer velocity increased to 20 mm/minute. Results were compared with one-way ANOVA and Student's *t*-test.

Incisions Treated with TBF-β in Vitrogen

To provide paired analysis, parallel incisions 10 cm in length were made through the panniculus carnosus 1 cm on either side of the dorsal midline of 18 adult male Sprague-Dawley rats (450 g). One incision of each rat was treated with 1 ml of Vitrogen collagen (Collagen Corporation, Palo Alto, CA), and the other incision was treated with 1 ml of Vitrogen collagen containing 2 μg of TGF-β. Incisions were closed with three evenly placed interrupted sutures of 3–0 Ethilon (Ethicon Inc., Somerville, NJ). At 5, 9, and 14 days after surgery, six rats were killed, and tensile strengths were measured as before. The two treatment groups were compared for statistical significance using one-way ANOVA and paired *t*-test analysis.

Results

Effect of a Single Dose of EGF-Saline on Tensile Strength

Utilizing a simple formulation for administering a single dose of EGF, incisions were treated with EGF dissolved in phosphate-buffered saline. The average tensile strength of incisions treated with EGF was not significantly different than incisions treated either with PBS or simply sutured at 10 days after surgery (2.81 ± 0.96 *vs.* 2.64 ± 0.69 *vs.* 2.84 ± 0.68 kg/cm^2, respectively). In the second experiment, tensile strength of incisions treated with a single dose of EGF formulated in hydrogel was not significantly different than incisions treated with hydrogel vehicle alone or untreated incisions at 7, 14, 21, or 28 days (Table 1).

Effect of Repeated Doses of EGF-Saline on Tensile Strength

EGF-treated incisions were an average of 35% stronger than saline-treated incisions 7 days after surgery ($p < 0.02$) (4.51 ± 1.54 *vs.* 3.33 ± 1.30 kg/cm^2). Fourteen days after surgery, the tensile strength of EGF-treated and saline-treated incisions were not different (6.23 ± 1.34 *vs.* 6.55 ± 1.87 kg/cm^2). Microscopically, the tract of the EGF-treated incision 7 days after surgery was very narrow with closely opposed edges. A plug of epidermal cells extended a short distance into the incision and then progressed into an intermittent string of cells. In comparison, the saline-treated incision was filled by a wide plug of epidermal cells extending the full length of the incision. The 14-day specimens showed no appreciable differences.

Stability of Encapsulated EGF

Since EGF formulated in PBS or water-miscible gel failed to increase tensile strength, we next developed formulations which would retain EGF in the area of incisions providing prolonged exposure of cells to EGF. Multilamellar liposomes containing ^{125}I-EGF were prepared, and their stability was determined by dialysis against saline or simulated interstitial fluid. After 14 days of dialysis against PBS at 37 C, 43% of the ^{125}I-EGF was lost from the liposomes, and at 4 C, 31% was lost. The rate of loss of radioactivity from the dialysis bags was linear for samples dialyzed at either 37 C or 4 C (correlation coefficient: at 37 C, r = 0.99; at 4 C, r = 0.99). After 14 days of dialysis against simulated interstitial fluid at 37 C, 67% of the liposomes remained intact as measured by either centrifugation or dialysis of the liposomes.

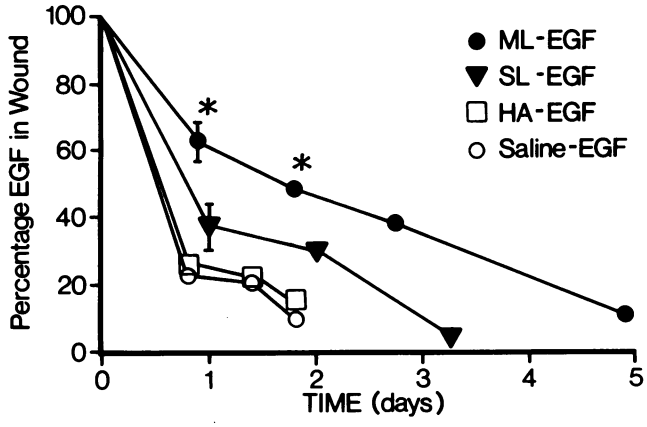


FIG. 1. Retention of EGF in incisions. Forty-eight adult male Sprague-Dawley rats were divided into four equal groups. One of four formulations containing ^{125}I-EGF and unlabeled insulin as carrier was then placed in the base of the incisions: multilamellar liposomes (ML-EGF) (●), single lamellar liposomes (SL-EGF) (▼), hyaluronic acid (HA-EGF) (□), saline (saline-EGF) (○). Values are the mean and standard deviation of three incisions. *$p < 0.001$ *vs.* HA-EGF and *vs.* saline-EGF.

Retention of EGF in Incisions

The EGF-liposome preparations demonstrated substantial stability *in vitro*. Therefore, we measured the rate of loss of EGF from incisions *in vivo* using EGF-liposomes. As shown in Figure 1, 22% of the ^{125}I-EGF formulated in saline and 23% of the ^{125}I-EGF formulated in 1% hyaluronic acid were retained in the incision 1 day after surgery, and about 16% was retained after 2 days. Slightly more ^{125}I-EGF was retained in incisions when formulated in single lamellar liposomes (37% after 1 day, dropping to 4% after 3 days). Significantly higher levels of ^{125}I-EGF were retained in incisions for extended periods by multilamellar liposomes entrapping EGF (62% after 1 day, 48% after 2 days, and 11% after 5 days).

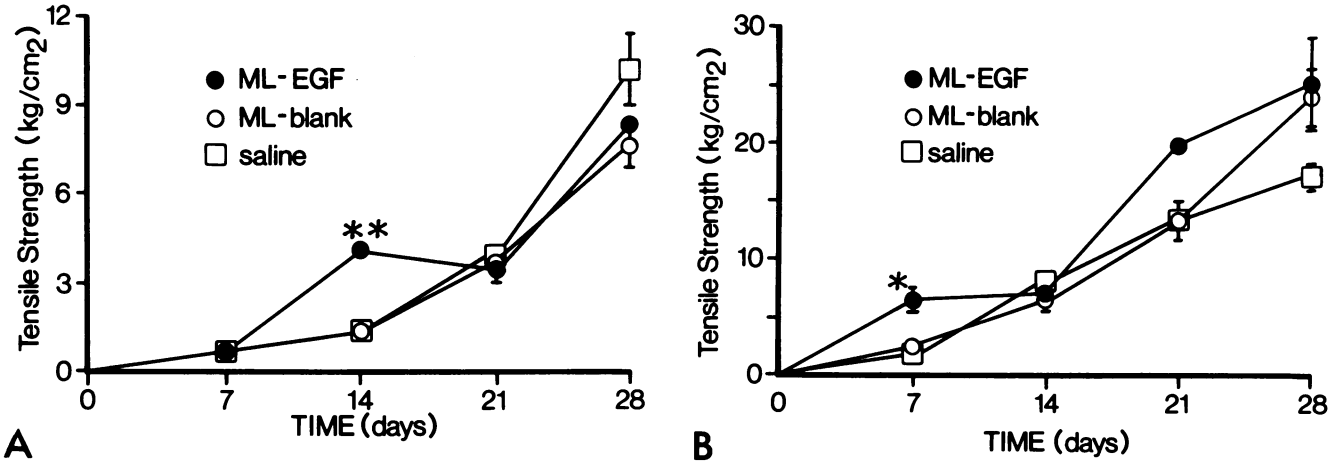
Effect of Liposome-Encapsulated EGF on Tensile Strength

Based on the superior retention of EGF in incisions when formulated in liposomes, we tested the effect of a single application of multilamellar liposomes containing EGF on tensile strength of incisions. In the first experiment (Fig. 2A), tensile strength of incisions treated with EGF-liposomes was three times greater than saline or blank liposome incisions (4.04 ± 0.36 *vs.* 1.42 ± 0.12 *vs.* 1.36 ± 0.11 kg/cm^2, respectively) 14 days after surgery ($p < 0.01$). Twenty-one days after surgery, tensile strengths of control incisions had increased to equal the strength of EGF-treated incisions. Twenty-eight days after surgery, tensile strengths of all three treatment groups continued to increase, and all groups had similar tensile strengths. No significant difference was observed between incisions treated with saline or blank liposomes at any point.

Light and electron microscopy of incisions 14 days after treatment with EGF-liposomes revealed a hypertrophic epidermal layer with reformation of an early basement membrane directly over the incision. Many active fibroblasts containing abundant rough endoplasmic reticulum were present in the incision, accompanied by new collagen formation. In comparison, untreated incisions or incisions treated with blank liposomes had substantially less hypertrophy of their epidermal layer with no basement membrane reformation. At the ultrastructural level, control incisions had less collagen remodeling and more tissue edema than the EGF-treated incision.

The 28-day specimen treated with EGF had the ultrastructural appearance of a very strong scar with well-formed, bonded, and complete collagen with sparse ground substance. Large numbers of very active fibroblasts were present in the incision tract. In contrast, fewer active fibroblasts were present in control incisions. In addition, remodeling of collagen was less advanced with considerable ground substance remaining, giving the incisions an edematous appearance.

In the second experiment (Fig. 2B), tensile strengths were measured at 7, 14, 21, and 28 days after surgery. As tensometer velocity was significantly increased, the results from the first and second experiment are not directly comparable. At 7 days, the average tensile strength for incisions treated with EGF entrapped in multilamellar liposomes was approximately threefold higher ($p < 0.05$) than tensile strengths for blank liposomes or saline-EGF incisions (6.45 ± 1.04 *vs.* 2.50 ± 0.10 *vs.* 1.95 ± 0.14 kg/cm^2, respectively). At 14, 21, and 28 days, tensile strengths of incisions treated with blank liposomes or saline-EGF were not significantly different than the strength of incisions treated with multilamellar-EGF liposomes.



FIGS. 2A and B. Tensile strength of incisions treated with EGF-liposomes. Each incision received 1 ml of liposome suspension containing 5 μg of EGF (ML-EGF) (●), an equal amount of blank liposomes (ML-blank) (○), or saline (saline) (□). In experiment 1 (A), tensometer rate was 5 mm/min and n = 9 measurements at each point. In experiment 2 (B), tensometer rate was 20 mm/min and n = 18 measurements at each point. Values are the mean and standard error of the means. **p < 0.01, *p < 0.05 *vs.* ML-blank and *vs.* saline.

Effect of Single Dose of TGF-β on Tensile Strength

Since TGF-β was reported to stimulate collagen synthesis,[4] we evaluated the effect of a single dose of TGF-β on tensile strength. As shown in Figure 3, wound tear strength of TGF-β-treated incisions was 51% greater than control incisions (2.56 ± 0.22 *vs.* 1.69 ± 0.19 kg/cm^2) at 9 days after surgery ($p < 0.01$). No significant differences in tensile strengths were measured at 5 or 14 days.

Discussion

Healing of incisions is a complex process involving epidermal regeneration, fibroblast proliferation, neovascularization, synthesis, and remodeling of extracellular matrix components.[19] EGF has been shown to stimulate mitosis of epidermal keratinocytes[20] and dermal fibroblasts[5] and to promote angiogenesis.[21] TGF-β has been shown to stimulate collagen synthesis in rat kidney fibroblasts.[4] Thus, EGF and TGF-β potentially could enhance wound healing resulting in accelerated gain of tensile strength in incisions. Results of the present study validate the "proof of principle" that treatment of exogenous growth factors delivered in appropriate vehicles increase tensile strength during the early phases of wound healing.

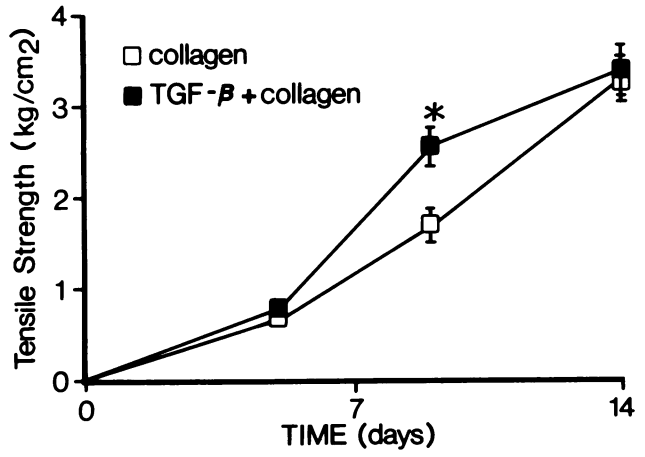


FIG. 3. Tensile strength of incisions treated with TGF-β in collagen. Two parallel, 5-cm incisions were made through the panniculus carnosus 1 cm on either side of the dorsal midline of 18 adult male Sprague-Dawley rats. One incision of each rat received 1 ml of collagen (□), and the other incision received 2 μg of TGF-β in 1 ml of collagen vehicle (TGF-collagen) (■). Values are the mean and standard error of the means and n = 18 measurements at each point. *p < 0.01 *vs.* collagen.

Previous *in vitro* experiments have clearly demonstrated that cells must be continuously exposed to EGF for 8–12 hours before they are stimulated to divide.[22,23] Thus, the vehicle used to deliver EGF to incisions is a key component. Our results demonstrate that EGF formulated in saline was very rapidly lost from incisions (80% lost in 24 hours) and did not increase tensile strength of incisions. However, a significant increase in tensile strength was produced when EGF formulated in saline was repeatedly injected near the incision for 5 days. Furthermore, an early increase in tensile strength was stimulated when a single dose of EGF was delivered in a delayed release formulation (multilamellar liposomes), such that there was prolonged exposure of growth factor to the wound.

A similar response was demonstrated by Buckley et al.[12] using polyvinyl alcohol sponges implanted subcutaneously in rats. Daily injections of 10 μg of EGF into the sponges promoted moderate formation of granulation tissue. However, when sponges were implanted with pellets, which continuously released 10 μg of EGF per day, increases of capillary ingrowth, DNA content, protein content, and wet weight were observed at 7 days. As with the present study, the effect was only transient as no differences were observed at 3 or 14 days between sponges

containing placebo and EGF-releasing pellets. Laato et al.[13] also reported that daily injections of 5 μg of EGF in PBS into cellulose sponges implanted subcutaneously on the backs of rats increased DNA and hydroxyproline content at 7 days. However, a single injection of EGF given immediately after wounding had no effect on the developing granulation tissue. Sporn et al.[24] also found that daily injections of EGF (20 ng) or TGF-β, alone or in combination with EGF, into stainless steel wound chambers on the back of rats stimulated substantial increases in protein content after 5 days. Grotendorst et al.[6] utilized stainless steel chambers prefilled with collagen gel containing EGF, insulin, or platelet-derived growth factor PDGF implanted under the skin on the back of rats. After 14 days, both EGF and PDGF, but not insulin, significantly increased new collagen accumulation compared with controls.

Results from other wound models provide additional evidence supporting the importance of prolonged continuous exposure of EGF to stimulate wound healing. Repeated topical treatment of partial-thickness burns with EGF accelerated epidermal regeneration when EGF was formulated in vehicles that prolong the exposure of residual epithelial cells to EGF.[10] Brief exposure of mid-dermal injuries to EGF formulated in saline failed to stimulate healing.[25-27] In addition, repeated applications of EGF induced hyperplastic regeneration of tympanic membrane perforations in cats (unpublished data) and increased tensile strength of corneal incisions.[9,11]

The mechanism of action of TGF-β is poorly understood and may not require continuous, prolonged exposure to induce biologic responses. For example, a single subcutaneous injection of TGF-β in saline induced fibrosis in rats,[4] and a single dose of TGF-β reversed the doxorubicin-induced impairment of thymidine uptake and hydroxyproline production in wound chambers in rats.[28] Our results demonstrate that a single dose of TGF-β stimulated early increase of tensile strength in rat incisions. Our results are in agreement with those of Mustoe et al.[29] However, the tensile strength of growth factor–treated incisions, expressed as a percentage of control, was similar to control incisions at the end of each sampling period (Fig. 4). We conclude that the increase in tensile strength stimulated by EGF and TGF-β is transient in normal animals. It is probable that the ability of exogenous growth factors to accelerate wound healing in healthy animals is due to earlier delivery or increased levels of growth factors than occurs naturally.

While no physiologic role for growth factors has been definitively established in normal wound healing, recent data imply that they may play important roles. In a recent clinical trial, biosynthetic human EGF accelerated epidermal regeneration of donor sites and chronic nonhealing diabetic ulcers (unpublished data). TGF-β levels rise transiently in rat wound chambers,[16] and several potent growth factors are present in platelet granules. An extract of platelets containing growth factor activity stimulated healing in chronic skin ulcers.[3] Localized deficiency of growth factor action may play important roles in a variety of impaired wound healing states.

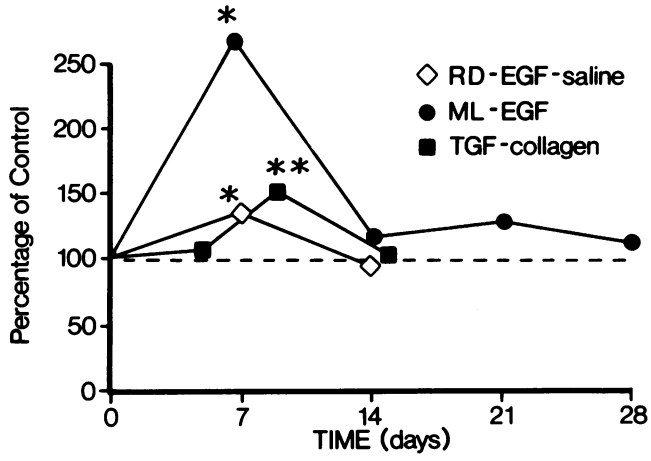


FIG. 4. Percentage tensile strength compared to controls. Mean tensile strength values for each growth factor treatment group were expressed as a percentage of mean control tensile strength values for each experiment. Repeated dosage EGF-saline (RD-EGF-saline) (◇); multilamellar liposome-EGF (ML-EGF) (●); TGF-β-collagen (TGF-collagen) (■). *p < 0.05, **p < 0.01 *vs.* each control incision.

In summary, the results presented here demonstrated that EGF and TGF-β accelerate early tensile strength gain of cutaneous incisions in normal rats. In addition, formulation of EGF in multilamellar liposomes provided prolonged, local delivery of EGF to the wound and increased early tensile strength. The current availability of growth factors through recombinant DNA technology and the possibility of clinical applications make them a potentially important adjunct to the surgeon. Additional studies need to be conducted to determine the effect of local application of growth factors in impaired wound healing models.

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